STUDIES OF THE PROCEDURES FOR THE ISOLATION OF SALMONELLAE FROM FOODS

by

SAMIR A. AL-DAHHAN

D.V.M., Baghdad University, Baghdad, Iraq, 1961

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1965

Approved by:

W. D. Foltz

Major Professor

TABLE OF CONTENTS

INTRODUCTION 1

LITERATURE REVIEW 2

Liquid Enrichment Media for Salmonellae 2

Differential Plating Media 6

Phage Typing Using Genus Specific O-1 Phage 8

Comparison Between Different Procedures 9

Isolation of Salmonella from Different Foods 12

MATERIALS AND METHODS 12

First Method (Nutrient Broth Pre-enrichment and Mannitol Purple Pre-enrichment) 12

Samples 12

Sampling 13

Pre-enrichment 14

Selective Enrichment 15

Differential Plating Media 15

Purification of Cultures 16

Phage Lysis 16

Serological Identification 16

Somatic Antigen 17

Flagellar "H" Antigen Analysis 18

Interpretations 19

Second Method (Tetrathionate Enrichment Method) 20

Third Method (Lactose Broth Pre-enrichment Method) 22

Fourth Method (Screen Test for Salmonellae Using the Lactose Pre-enrichment Technique) 23

Fifth Method (Selenite Cystine Enrichment) 23

Sixth Method (Lactose Broth Pre-enrichment Using Brilliant Green Tetrathionate) 25

RESULTS 26

Effect of Tergitol No. 7 35

Reactions in Triple Sugar Iron Agar or Kligler Iron Agar 36

Results on Phage Lysis 36

Results on Serology 36

DISCUSSION 40

SUMMARY 48

ACKNOWLEDGMENTS 50

BIBLIOGRAPHY 51

APPENDIX 58

INTRODUCTION

Salmonellae and salmonellosis have always been an interesting subject for those involved in medical, public health, and food research, especially since salmonellosis has been proved to be a serious problem for both humans and animals (Akiyama, 1961; Essex et al., 1963; De Capito, 1963; Francis, 1963; Frankl and Rogers, 1961; Richardson, 1961; Stephens, 1963; Triozon, 1963; Vinogradov, 1961), Akopiau (1961), and Angelloti (1901).

New Salmonella serotypes are always being discovered, most of which are pathogenic to humans (Levin, 1961; Akopian, 1960; Sachideva, 1963; Ellingsen, 1962; Angelotti, 1961; Pollo and Mellande, 1960; Lerche, 1961; and Haas, 1962; Brede, 1963; and Guinee, 1961).

The above mentioned facts and the international scope of salmonellae problems (LeNoc, 1963; Muller, 1957; Marcus, 1963; Richardson, 1961; Thomas, 1961; Vinogradov, 1961; and Khakareva, 1963) have naturally resulted in workers all over the world devoting years of research work for the invention and improvement of the best methods for detection and isolation of salmonellae from food products. A review of literature revealed that not only very few attempts have been made to compare various methods commonly employed in salmonellae detection, but that many of these methods have not been adequately proven in effectiveness and were originally meant for detection of salmonellae in clinical specimens collected from carriers. The methods were assumed to be as effective in food work as in clinical practice, which was unfortunately not so (McCullough and Byrne, 1952).

Therefore, the following study was an attempt to compare some of the most common methods in use for detection and isolation of salmonellae from naturally contaminated foodstuffs, and to suggest the best method for accomplishing this task.

LITERATURE REVIEW

Members of the genus Salmonella were first recognized in the late 19th century and were considered as the wide-spread cause of human and animal infection. Numerous methods for the isolation of these organisms, mainly from clinical specimens (Van Eck, 1961; and Martin, 1961) have been developed; but, further work eventually showed that foodstuffs played an important part in the chain of infection. The same methods used for examination of clinical specimens were first employed, but they were proven to be inadequate in many instances (Dack, 1956), because the methods that are good for clinical specimens are not necessarily so for food samples. For example, the addition of comparatively large amounts of organic material, such as food, may have an adverse effect on the selectivity and the enrichment quality of these media (Dack, 1956).

Liquid Enrichment Media for Salmonellae

The two selective liquid enrichment media that had been used most widely are the tetrathionate broth of Muller as modified by Kauffman, and the selenite broth of Leifson (1936). However, Thomson (1955) found that ordinary nutrient broth was often as effective as, or even superior to, selenite broth for the enrichment of salmonellae from feces. Smith

(1952, 1959b) found that direct plating of intestinal contents on a selective or differential agar was superior to selenite and tetrathionate enrichment for the isolation of Salmonella cholerae-suis and S. abortus-ovis and later recommended brilliant green - MacConkey broth for the isolation of the former type. He concluded that the selenite and tetrathionate broths were toxic for these types.

Banwart and Ayres (1953) found that tetrathionate broth inhibited the growth of S. paratyphi "A"; therefore, several modifications of tetrathionate broth have been recommended to suppress the growth of Proteus. Galton et al. (1962) found that addition of 0.125 mg of sodium sulfathiazole per 100 ml suppressed the multiplication of Proteus from canine fecal swabs. Jameson (1961) reported that the addition of 1% sodium lauryl sulfate and bismuth sulfite solution to tetrathionate broth favored the selective inhibition of Proteus and allowed active multiplication of salmonellae from sewer swabs.

During work on food samples, Galton et al. (1954) found that the addition of wetting agent "Tergitol No. 7" (sodium heptadecyl sulphate) to tetrathionate broth helped in dispersion and emulsification of the heavy layer of fat on the broth. Later Galton et al. (1955) found that while Tergitol appeared to enhance growth of salmonellae within a wide range of dilutions, it was also found helpful in the rehydration of dried dog meals.

Osborne and Stokes (1955), North and Bartram (1953), and Stokes and Osborne (1955) worked on selenite broth and modified it by the addition of cystine then sulfapyridine. Byrne et al. (1955) found

that rehydration of dried eggs and egg products in distilled water and inoculation of this diluted suspension into multiple tubes of selenite cystine broth favored the isolation of salmonellae. Similar enhancement of growth was obtained from dried yeast when an aqueous suspension was incubated at 30 C for 24 hours prior to inoculation of selenite or tetrathionate broths. Slocum (1955) found that the use of pre-enrichment media before the use of selective media was better than modification of the composition of the latter.

North (1960) reported the effective enhancement of growth due to the use of lactose broth pre-enrichment medium, especially on samples containing small numbers of salmonellae. He explained it as due to the restoration of a larger number of salmonellae that have been in a state of dormancy due to the processing of food, like freezing, drying, etc. Taylor and Silliker (1958) used tricarboxylic acid metabolites for pre-enrichment of food known to contain salmonellae with the object of re-juvenating the damaged cells, but to no avail.

The inhibitory effect of albumen (egg white) on the performance of selective media was earlier studied by Taylor and Silliker (1958).

Thomson (1955) was able to isolate S. paratyphi "B" from a sample of flour for nearly a year when it was inoculated into nutrient broth medium, whereas, cultures from the same sample were negative after 5 months when tested with selenite broth. He also observed that inoculation of small amounts of samples was better than large amounts. He noticed that if a feces sample was diluted up to 1:1000, coliforms progressively decreased, giving a better change for salmonellae colonies to

grow on selective plating media. He thought that the advantage of selenite and tetrathionate media was generally due to their fluid nature rather than their composition.

Other workers attributed better results in higher dilutions due to the probability of the presence of salmonellae in clumps, and the dispersion of these clumps during dilution.

Dack (1956) found that salmonellae could be recovered more effectively from egg albumen by inoculation into non-inhibitory broth (lauryl tryptose or nutrient broth) containing polyvalent Salmonella "H" antisera. After incubation and centrifugation, the sediment was incubated in selenite cystine broth.

Jansen (1955) suggested the use of both tetrathionate and selenite broth for enrichment to obtain better recovery.

Wells et al. (1957) confirmed the value of their brilliant green sulfapyridine selenite broth suggested by Osborne and Stokes for screening egg products.

Silliker and Taylor (1958) noted that the presence of gelatine and albumen in food materials markedly diminished the performance of enrichment broths. The use of centrifugation for separating the bacteria from soluble food materials improved the function of enrichment media. They observed that the preponderance of coliforms did not affect the selectivity of either selenite or tetrathionate broths.

Taylor and Silliker (1958) compared selenite-cystine "F" medium with a modified selenite cystine medium in which mannitol and dulcitol were substituted in place of lactose, while using samples containing both

coliforms and salmonellae. Selenite brilliant green sulfapyridine was also used simultaneously. But all these modifications failed to give any better results.

Taylor and Silliker (1958) also established that reconstitution of albumen in distilled water prior to inoculation into enrichment media was better than direct transfer. But lactose broth pre-enrichment was superior to reconstitution in distilled water and that the type of carbohydrate added to the pre-enrichment broth was not significant; dulcitol, mannitol and lactose gave comparable results.

Differential Plating Media

For general isolation of salmonellae and other enteric pathogens, many plating media have been developed (such as Endo's agar and eosin-methylene blue agar) on which lactose and non-lactose fermenting organisms may be distinguished and Gram positive organisms are inhibited.

Certain other media, like MacConkey's (M.A.C.) agar and Leifson's desoxycholate agar, contain bile salts which make them selective for Gram negative bacteria, and at the same time suppress the spreading of Proteus. However, highly selective plating media are more effective for the isolation of salmonellae by direct plating or by plating from enrichment broths, because they are designed to inhibit many strains of the normal intestinal flora.

The most widely used selective media are bismuth sulfite agar (B.S.A.), desoxycholate citrate agar (D.C.A.), S.S. agar, MacConkey's agar, brilliant green agar (B.G.A.), and brilliant green phenol red

agar. Salmonellae can be isolated on all these media, but the brilliant green agar plates are more inhibitory for enteric organisms other than salmonellae, besides the ease with thich salmonellae colonies are detected on it.

Galton et al. (1954) noted that in examination of meat, and other materials in which Pseudomonas species were in large numbers, the addition of 8 to 16 mg of sodium sulfadiazine per 100 ml of brilliant green agar gave excellent results. While pseudomonads were effectively inhibited, there was no inhibition of salmonellae.

Osborne and Stokes (1955) found that addition of 0.1% sulfapyridine to brilliant green agar enhanced its selectivity for salmonellae, especially in examination of egg products. All the above media are described in detail in the Difco Manual (1953) with their specific uses and advantages.

Banwart and Ayres (1953) noted that S. paratyphi, S. typhimurium, S. oranienburg, S. pullorum, S. anatis and S. worthington grew luxuriantly on brilliant green agar. They also noticed that B.S.A. and S.S. agar were highly inhibitory for these organisms. Byrne et al. (1955) used B.S.A. and B.G.A. in a S. pullorum study. B.G.A. was inhibitory to this organism while B.S.A. was not. They felt that no single plating medium would suffice, and the employment of many media would certainly increase the chances of recovery.

Taylor et al. (1958) noticed that B.G.A. was superior to S.S. and B.S.A. because distinct differentiation could be made easily between coliforms and non-coliforms in about 24 hours incubation.

Edwards and Ewing (1962) recommended the use of B.G.A. for isolation of salmonellae other than S. typhi.

Phage Typing Using Genus Specific O-1 Phage

Cherry et al. (1954) used this phage for identification of salmonellae from other Gram negative organisms. The phage was tested with a large number of salmonellae strains, Shigella, Proteus, Serratia, Escherichia, Aerobacter, Bethesda, monophasic Arizona and diphasic Arizona and 95 other strains of miscellaneous organisms. They found that with the rare exception one strain of Escherichia, one strain of Shigella, and one miscellaneous strain, only Salmonella and diphasic Arizona strains could be lysed by this phage.

The use of B.G.A. on which to show the lysis was suggested because it permitted easy recognition of lactose fermenters as well as providing a good contrast in background for reading of lysis results. Sometimes strains of S. derby, S. anatum, S. newington, S. pullorum, S. tennessee and S. senftenberg were fairly resistant to phage lysis.

Quite recently much work has been done on this aspect of Salmonella identification. Milch and Laizlo (1963) typed a total of 1,895 S. typhimurium strains by Felix and Callow's phage and found it to be of great epidemiologic value. Dung and Kang (1963) studied 138 strains of S. cholerae-suis to determine their epidemiologic value, which was found to be quite significant.

Kilesso (1963) has done similar work with Salmonella "O" bacteriophage.

Other detailed studies have been done on the bacteriophage of S. paratyphi "A" (Joshi, 1963) and on S. typhi-murium by Sharma (1963) which the reader is referred to.

Comparison Between Different Procedures

A review of the literature revealed the lack of such work; however, recently Taylor et al. (1964) worked on a comparison of two methods, comparing 179 meat and egg samples using: (1) the Food Hygiene laboratory of England procedure which is a direct enrichment method, and (2) the pre-enrichment method devised for processed food in the United States of America. The number of positive samples and replications, and the number of strains and kinds of serotypes were statistically comparable by both methods. Boneless frozen beef, veal and horsemeat imported from five countries for consumption in England were found to have salmonellae present in 48 of 116 samples (i.e., 41%). Dried egg products imported from three countries were observed to have 16% of the samples positive. They also found that two or more different strains of salmonellae could be detected in one sample by using different methods, and that the total number of positives from the two methods was observed to be greater than the number of positives from any one method.

However, a slightly higher percentage of salmonellae isolations was shown by the Food Hygiene Laboratory method.

Silliker et al. (1964) worked on the comparison of methods for the isolation of salmonellae from egg products using three different enrichment procedures, viz: selenite broth, selenite broth containing 10% sterile feces, and lactose broth pre-enrichment procedure.

They found that although the lactose pre-enrichment method promoted salmonellae recovery from samples containing small numbers of dormant organisms, the efficiency of this method was adversely affected by unfavorable coliform-salmonellae ratios. They suggested that under such conditions, early subculture of lactose broth into the selenite broth should be done. Selenite broth containing 10% sterile feces was more efficient than the lactose pre-enrichment methodology in promoting the growth of dormant salmonellae. They also noted that albumen adversely affected recovery of salmonellae from selenite broth, whereas whole eggs and egg yolks enhanced recovery of salmonellae from this medium. It is their opinion that selenite feces medium presents a solution to the major problems encountered in the detection of salmonellae in egg products, and offers an approach to a single medium in which food-borne salmonellae will manifest themselves with a minimum of laboratory manipulation.

Earlier, Taylor and Silliker (1961), on comparing several methods for isolation of salmonellae by enhancing the growth, revealed that inoculation of a second enrichment broth from the first was no improvement over the single direct enrichment method. It was inferior to centrifugation.

Selenite was observed to produce more positive isolations at 48 hours than at 24 hours. No change occurred in tetrathionate. Reconstitution of dried albumen with water produced a significant increase in isolation over direct inoculation of enrichment broth in the case of tetrathionate but not selenite broth.

Pre-enrichment in lactose broth before inoculation of enrichment media was vastly superior to reconstitution in water for both enrichment broths. A comparison of results obtained using dulcitol, mannitol, lactose and carbohydrate-free purple broths in pre-enrichment indicated that the carbohydrate added was immaterial.

In the same year as the comparisons by Taylor and Silliker (1961), Montford and Thatcher (1961) compared three methods by which salmonellae may be isolated and enumerated from dried albumen, e.g., direct inoculation of enrichment media; centrifugation of samples and inoculation into pre-enrichment media; and centrifugation of samples and pre-enrichment in non-inhibiting media. The results of the study revealed the pre-enrichment to be the method of choice. The superiority of pre-enrichment manifests itself in replicate aliquots of the same sample by producing a statistically significant increase in numbers of isolation of salmonellae and in empirical use with various albumen samples by consistently higher values of most probable numbers (M.P.N.). They also indicated that the primary factor involved in this superiority appears to be the greater ability of small numbers of salmonellae to initiate growth in the non-selective amnnitol purple sugar broth than in the inhibitory enrichment media.

They concluded that the method of analysis recommended entails inoculation of mannitol broth pre-enrichment medium, transfer of 24-hour culture aliquots into tetrathionate broth and streaking on brilliant green agar for isolation of salmonellae.

Isolation of Salmonella from Different Foods

In Canada eggs and egg powder were found to be responsible for occurrence of salmonellae. Gibbons and Moore (1944), Ratna and Dolman (1948), and Thomson (1955) found S. paratyphi "B" in wheat flour.

Recently Wilson (1961) and Wright and Anderson (1962) found salmonellae in meat and poultry products and other food additives of animal origin, respectively. Lerche (1961) studied some salmonellae isolations from mayonnaise.

Thatcher and Montford (1962) isolated salmonellae from a large variety of food samples, like frozen eggs, and cake mixes with and without eggs.

MATERIALS AND METHODS

First Method* (Nutrient Broth Pre-enrichment and Mannitol Purple Pre-enrichment)

Samples. All the samples that were canned, packaged, or sealed were purchased from different local stores in Manhattan, Kansas. The samples included cake mixes, cookie doughs, biscuits, dinner rolls, and pie mixes. The remainder of the samples, which included different kinds of eggs, corn meals, tankage, horse feed supplement, and milk replacer, were obtained from different places within a 50-mile radius of Manhattan.

---

* The first method consists of two separate procedures and is considered as two separate methods in the text.

The frozen samples were immediately put in the freezer. The moist samples were also put under refrigeration of 6 to 8 C. The dry samples were stored on shelves at room temperature until the time of examination, which did not exceed one week following collection of the samples.

The total number of the samples collected was 205. The collection included: 92 cake mixes, 48 eggs (frozen, liquid, and dry), 30 cookie doughs, 14 biscuit doughs, 10 corn meals, 6 pie mixes, 2 dinner roll doughs, 1 tankage, 1 milk replacer, and 1 horse feed supplement.

Sampling. Every possible precaution was taken to avoid contamination from outside.

The packages of cake mixes were wiped with alcohol, then opened with a flamed spatula; cookie dough, rolls, and biscuit dough containers were opened with a flamed knife after wiping them with alcohol. The bottle or jar mouths were flamed in the case of the egg samples. In every case of the above, sterile wooden tongue depressers were used, and materials were taken from the middle of each container and transferred into the medium in which was put in a sterile, screw capped, flat-bottomed bottle (4" x 2") of 250-ml capacity. The required amount of the samples was measured directly into the sample bottles by the use of a scale.

The samples were aseptically sealed again with tape and stored for further use or reference, at the proper temperature needed for each of them.

This method of sampling was followed in all of the procedures and experiments described in this paper.

The testing procedure included the use of pre-enrichment media, selective enrichment media, differential plating media, then final identification on Kligler iron agar, or triple sugar iron agar. Each of these steps will be described in detail.

Pre-enrichment. In this method, two pre-enrichment media were used:

1. Nutrient broth with the following composition:

Proteose peptone No. 3 (Difco)	10 grams
Beef extract (Difco)	3 grams
Yeast extract (Difco)	3 grams
Sodium chloride	5 grams

All ingredients were dissolved in distilled water (1000 ml) by boiling. The pH was adjusted to 7.6 by normal sodium hydroxide solution.

2. Mannitol purple broth was prepared by adding 0.5% of mannitol to purple broth base (Difco) dissolved in 1000 ml distilled water.

Both media were autoclaved at 121 C for 15 minutes after 90 ml of the medium had been distributed into 250-ml bottles.

As a further precaution the bottles were incubated at 37 C for 24 hours, and only those showing no growth or turbidity were used.

In this method 10 gms of the sample were added with the previously mentioned aseptic precautions, into the bottles containing the media, tongue depressers were used first for transferring the material, then for stirring the sample with the medium to make a homogeneous suspension. The bottles were then incubated for 8 hours at 37 C. At the end of this period 1 ml of each of the samples was transferred aseptically into tubes with 15 ml of selective enrichment media.

Selective Enrichment. Modified tetrathionate broth (Difco) and selenite broth "F" (Difco) were used in transferring from nutrient broth pre-enrichment. They were modified by the addition of cystine and sulfapyridine 100 mg and 500 mg per liter of the medium, respectively, to selenite "F" tubes, and by the addition of brilliant green to tetrathionate tubes, in amounts sufficient to give a final concentration of 1:100,000. The tubes were then incubated at 37 C for 24 hours, then streaked on three kinds of differential plating media.

Differential Plating Media. The following plating media were used:

1. Brilliant green agar (Difco) (B.G.A.)
2. MacConkey agar (Difco) (M.A.C.)
3. Bismuth sulfite agar (Difco) (B.S.A.)

The first two media were rehydrated, according to the manufacturer's specifications, distributed in 100 ml amounts into 6-oz prescription bottles, autoclaved at 121 C for 15 min, and left to solidify. When they were to be poured into plates, they were re-melted.

The last medium was not supposed to be autoclaved, so it was poured into plates and left to solidify immediately after preparation.

All the plates were left partially covered for about an hour to ensure a dry, solid surface before streaking.

After streaking, the plates from each of the selective enrichment tubes were incubated for 24 hours to make an additional isolation if necessary. At the end of the first 24 hours the center of each of three suspicious colonies was picked from each plate and transferred into Kligler's iron agar (K.I.A.) by stabbing the bottom then streaking the slant, followed by incubation at 37 C for 12 to 24 hours.

Identification was done by checking for the changes that took place in the medium, which included hydrogen sulfide positive reactions and hydrogen sulfide negative reactions.

Purification of Cultures. The growth on K.I.A. was suspended in about 5 ml of nutrient broth, then restreaked onto MacConkey agar plates, incubated for 24 hours, suspicious colonies were picked and inoculated again into K.I.A. (later triple sugar iron agar was substituted for the K.I.A.), incubated for 24 hours, then the changes were checked to see if they were identical to the original tubes.

Phage Lysis. Genus specific Salmonella O-1 phage obtained from Dr. Cherry of the Communicable Disease Center, Atlanta, Georgia, was used.

A loopful of the growth on K.I.A. tubes was emulsified in about 5 ml of sterile nutrient broth and incubated at 37 C and checked every hour for turbidity. Once a slight turbidity developed, the tubes were removed from the incubator and a loopful of the young culture was spread on a marked 1 cm^2 area of a brilliant green agar plate, left to dry for 5 to 10 minutes. A drop of the genus specific Salmonella phage was carefully placed on the culture spot from the tip of a sterile Pasteur pipette. The plates were incubated at 37 C and read at the end of 6 hours then confirmed at the end of 24 hours.

The positive reaction is represented by areas that look like a half moon made of growth, or a full moon of growth-free agar surrounded by a circle of clear luxuriant growth, depending on how accurately the phage drop was placed over the culture spot.

Serological Identification. All the phage positive cultures were subjected to antigenic analysis using both "O" and "H" (Difco) Salmonella typing antisera, for further confirmation.

Somatic Antigen. Each of the K.I.A. cultures was seeded on the surface of proteose peptone agar slants, incubated for 18 to 24 hours, and checked with Gram stained smears for confirmation of purity.

After incubation the growth on the slants was scraped off and emulsified in about 1 ml of 95% ethanol in small test tubes then heated in a water bath at 60 C for one hour, after which they were centrifuged at 1800 rpm for 10 min.

The supernatants were carefully discarded, and the sediments were left to drain in the tubes in an inverted position over a piece of filter paper in a test tube rack. Four to five hours later, the sediments were reconstituted with 0.5 ml of sterile normal saline, and by using a sterile pipette, the cells were uniformly mixed to form a stable, uniform suspension.

When the suspensions were ready, slide agglutinations were carried out. Clean, alcohol-washed, microscope slides were used. The slides were marked in equal halves with a crayon. A big loopful of the saline culture suspension was placed on each half of the slide. A drop of 0.05 ml of the antiserum was added using a sterile loop, quickly mixed with a toothpick and heated very gently over a flame, rocking the slide meanwhile to hasten the reaction, but avoiding excessive evaporation. The other drop of suspension was left undisturbed to serve as a control.

By the aid of a table lamp, the results were read. Commencing with poly-A-1, the groups, namely A, B, C_1, C_2, D, E_1, E_2, E_4, G. H. I and "V_1" were successively used one at a time. A positive reaction took place within 10 to 30 seconds, consisting of clumps of cells distributed

in fairly good-sized masses, with clear areas in between them. Any delayed or partial agglutination was considered negative.

If the cultures reacted with "Poly" but did not react with specific Salmonella "O" antisera groups, they were checked with Salmonella "V_i" antiserum, and if there was no reaction with the "V_i" antiserum, the cultures were considered as not of the Salmonella genus.

Flagellar "H" Antigen Analysis. For a final identification of the Salmonella species within a group as determined by "O" antisera, it was necessary to determine the "H" antigens and the phase of the organism at hand. The tube test of Edwards and Brunner (1947) was carried out: sterile 10-ml tubes of nutrient broth were inoculated from the stock culture under study and incubated overnight at 37 C. These were then inactivated by using equal volumes of 0.6% formalized physiological saline solution (6 ml formalin plus 8.5 gm sodium chloride in 1 liter of distilled water). The antigen was now ready for use.

One-tenth ml of the antiserum was diluted with 3.3 ml of sterile normal saline. The various diluted flagellar antisera were measured in 0.5 ml quantities in separate agglutination racks. The formalinized cultures were added serially in each set in 0.5 ml quantities. Immediately after the addition, the tubes were rocked vigorously. The racks were set in a water bath at 50 C for half an hour. Every 5 min the racks were gently lifted and checked for evidence of agglutination. In positive cases a fluffy, woolly mass developed which later settled to the bottom in a coarse mass.

The slide agglutination was also carried out in a similar way as in the case of the "O" antigens, using the above described formalinized

antigens, thus determining phase "1" and phase "2". At this stage undiluted (Difco) Bacto Salmonella "H" antisera of fractions a, b, c, d, eh, G complex, i, K, L complex, v, y, s, z_4, I complex, z_{10}, z_{29} and en complex were employed.

Agglutination was complete in about 20 sec and no cross reactions were observed.

Interpretations. The results were compared with the Kauffman-White Schema (furnished by Edwards and Ewing, 1962) under the positive "O" group with which the test organisms had reacted. Then the results of the "H" antigen of organisms within the group were read to identify the organisms.

Before sending the cultures suspected to be salmonellae to the Kansas State Department of Health, Division of Public Health Laboratories, Topeka, Kansas, the rapid urea test was used at the beginning to rule out the possibilities of a Proteus culture.

The urea medium consisted of the following: urea, 2 gr; monopotassium dihydrogen phosphate, 0.1 gr; dibasic sodium phosphate, 0.1 gr; sodium chloride, 0.5 gr; ethyl alcohol, 1.0 gr; and distilled water, 99.0 ml.

The medium was adjusted to pH 7.0 with NaOH, and 0.5 ml of 0.2% aqueous phenol red was added.

This medium is not to be sterilized unless by filtration. It could be stored in the refrigerator for weeks without spoilage. But if the medium becomes pink (alkaline to phenol red), it should be discarded.

To perform the test, 0.2 ml amounts of the broth was distributed into tubes, marked properly, then heavily inoculated with generous amounts

of the growth from a triple sugar iron agar slant using a wire loop. The tubes were then incubated for one-half hour at 37 C then read. The change in color from pale yellow of the fresh medium to an intense pink or fuschia indicates the hydrolysis of urea, or a positive test.

Second Method
(Tetrathionate Enrichment Method)

Thirty gm of sample were weighed aseptically into screw-capped, sterile bottles containing 100 ml of tetrathionate medium prepared in the following way:

"Bacto" tetrathionate broth base was rehydrated according to the manufactuer's specifications. The medium was cooled and 1 ml of 1:1000 aqueous solution of brilliant green was added to 100 ml of broth, and 2 ml of iodine solution was added just prior to tubing. The iodine solution was prepared by dissolving 6 gm of iodine crystals (c.p. resublimed). Comparative studies have shown that the iodine solution may be added when the medium is prepared if used within 8 days after preparation (Edwards and Ewing, 1962). Six ml of a 10% aqueous solution of Tergitol No. 7, sterilized at 121 C for 20 min was added at that time, especially if the sample contained fat which might have interfered with the dispersion of the sample or with the removal of a loopful for further inoculation. The lids were tightened and the samples were shaken vigorously. Samples of dried products received a second shaking every one to two hours. Incubation was done at 37 C for overnight.

Brilliant green agar plates were prepared in the following way: 1.6% solution of sodium sulfadiazine in distilled water was prepared and boiled

for 10 min to sterilize, then 5 ml of this solution was added aseptically to each liter of brilliant green agar just prior to pouring off the plates. Each 100 ml of brilliant green agar contained 8 mg of sodium sulfadiazine.

After the plates were solid and completely dry, a generous loopful of the cultures was streaked on each of the B.G.S. (brilliant green sulfadiazine) plates and MacConkey plates. A 1:1000 dilution of the mixtures could be used in case of an expected overgrowth of salmonellae by other organisms. The plates were incubated at 37 C overnight.

Slants of T.S.I. (triple sugar iron) agar were prepared in such a manner as to obtain a one to one and one-quarter inch button. After sterilization in the autoclave and solidification of the slants, three Salmonella-like colonies were picked from each plate and stabbed then streaked on the T.S.I. slants, which were marked properly. The slants were then incubated overnight at 37 C. In picking up the colonies, care was taken to touch only the center of the desired colony, since organisms which are inhibited may be viable, but not visible as colonies.

Reactions typical of salmonellae were checked, and those proven positive were phage lysed on brilliant green agar plates in the same manner used in the first method. If the reaction was positive, the culture was examined by the slide test with polyvalent Salmonella antisera, and those showing definite Salmonella groups were sent to the Kansas Board of Health Laboratory for further confirmation as in the first method.

Third Method
(Lactose Broth Pre-enrichment Method)

Lactose broth medium (A.P.H.A., 1955) was prepared as follows:

Proteose peptone No. 3 (Difco), 10 gm; beef extract (Difco), 3 gm; yeast extract (Difco), 3 gm; Bacto lactose (Difco), 5 gm; and sodium chloride, 5 gm. The ingredients were dissolved in 1000 ml of distilled water and heated to boiling to ensure uniform distribution. The pH was adjusted to 7.0 by normal NaOH solution. The medium was distributed in bottles of the same capacity used in the first method. Some of the bottles had 126 ml in them while others had 90 ml. For each sample one 126-ml bottle, and two 90-ml bottles were used. To the first one, 14 gm of the sample was added and to the other two, 10 gm each of the sample was added to them. Using aseptic precautions, the mixtures were stirred carefully with sterile glass rods while adding the medium very slowly.

At this point the pH was checked again and adjusted to 7.0 by normal NaOH solution because some of the samples (especially eggs) may produce a very acidic reaction, due to fermentation. This reaction may be below the optimum pH for the growth of salmonellae. From the 14 gm samples, three 10 ml, three 1 ml and three 0.1 ml portions were transferred to tubes containing 10 ml of sterile lactose broth; this provided 3 jars containing 10 gm of egg or sample, two original jars and the remainder of the 14 gm jars. The tubes and jars were incubated at 37 C for 24 hours. A loopful of each of the jars and tubes was transferred into tubes containing 1 ml of selenite cystine broth which were incubated for 6 to 7 hours at 37 C. Each of the tubes of selenite cystine medium was

streaked on S.S. plates and B.G.A. plates and then was incubated overnight at 37 C. Suspected salmonellae colonies were picked into T.S.I. slants. Phage lysis, serological, and biochemical examinations were carried on to ensure and confirm the isolation as described in the first method.

Fourth Method
(Screen Test for Salmonellae Using the Lactose Pre-enrichment Technique)

Aseptically 25 gm of the samples was added to 100 ml of sterile lactose broth in jars, mixed thoroughly and incubated overnight at 37 C. Two jars each containing 100 ml of fresh, sterile selenite cystine broth were prepared. To one of them 10 ml of the lactose broth culture was added, and to the other, 1 ml of the same culture was added. These were in turn incubated at 37 C overnight. Plates of B.G.A. and S.S. agar were streaked from both dilutions and incubated for 24 hours at 37 C. Suspected colonies were picked and transferred to T.S.I. slants, and incubated for 24 hours at 37 C. Phage lysis, serological and biochemical tests were carried on as described in Method I.

Any positives by this method were checked by the North method for confirmation.

Fifth Method
(Selenite Cystine Enrichment)

Bacto selenite cystine broth, A.P.H.A. standard method formula, 1960, of the following composition was used: Bacto tryptose, 5 gm; Bacto

lactose, 4 gm; disodium phosphate, 10 gm; sodium acid selenite, 4 gm; cystine, 0.01 gm; and distilled water, 1000 ml. The medium was dissolved in the distilled water, then heated in flowing steam for 15 min, distributed in 100 ml amounts into sterile, screw-capped bottles. (It should not be autoclaved.)

Using the same precautions, followed in the first method, 10 gm of the samples were aseptically weighed into the selenite cystine broth bottles, mixed thoroughly with sterile tongue depressers, and incubated overnight at 37 C.

A big loopful of each culture was transferred to each of G.G. and MacConkey agar plates, and streaked on the dry surface of properly marked plates. The plates were incubated for 24 hrs at 37 C.

Three salmonellae-like suspected colonies were picked from each plate and transferred into T.S.I. agar slants taking care to cool the needle on the side of the agar before picking the colonies. The slants were stabbed, then streaked as usual and incubated at 37 C overnight.

Positive slants were treated as in the other methods, i.e., phage lysis, serological, and biochemical tests.

With a number of selected known positive and suspected samples, the same procedure was repeated except for incubation of selenite broth cultures for 48 hours instead of 24 hours and the results recorded, and compared with the 24 hours incubation batch.

Sixth Method
(Lactose Broth Pre-enrichment Using
Brilliant Green Tetrathionate)

This is primarily the same as the previous method except for the use of tetrathionate enrichment medium, described in Method II (Garton et al., 1964), and some other modifications in the lactose broth pre-enrichment use.

As aseptically as possible, 11.1 gm were weighed into sterile jars containing 99.9 ml of lactose broth containing 0.6% Tergitol No. 7, then the contents were thoroughly mixed. From each of these jars 10 ml and 1 ml amounts were transferred into tubes containing 10 ml each of lactose broth. Thus with the first jars and the tubes each sample provided 10 gm, 1 gm and 0.1 gm portions of the sample, in standard amounts of the medium.

All the samples were then incubated for 48 hours at 37 C after which 1 ml from each tube of the lactose broth was transferred into 10-ml tubes of tetrathionate enrichment medium, and incubated for 24 hours at 37 C at the end of which a loopful from each tube was streaked on B.G. agar and S.S. agar plates, incubated for 24 hours at 37 C, then three suspected colonies were picked from each plate into T.S.I. slants, and proceeded with the rest as usual.

A special experiment was performed using both direct enrichment methods on ten selected samples (5 of them known positive and 5 suspected). Equal amounts of the samples were put into two different sets of jars, one set with Tergitol No. 7 added, and the other without. A comparison was made using the results obtained on the tubes, and plates of differential selective media.

RESULTS

The work on this problem was done in two main stages.

A total of 205 samples of different kinds of foodstuffs was examined, employing the seven methods of isolation and identification of salmonellae outlined above. Ten of these samples were proven to be positive for salmonellae, and their serotypes were identified.

The nature of the sample and the number of positives isolated from each of the sample groups is presented in Table 1.

The new positive samples and a number of old known positive samples were re-examined and the same seven methods were employed using the data, observations, and information gained as a means of comparison between these methods.

In addition, other experiments were done like the experiment on the length of time needed for the incubation of selenite cystine cultures, where the 48 hours incubation was found to provide more recoveries than 24 hours.

The effect of Tergitol was tested on 10 samples (one with Tergitol and another without). No significant difference was noticed.

Of the 92 cake mixes examined, 59 samples contained eggs as one of the ingredients; the rest of the samples did not. Four of the positive samples contained eggs, and three of them did not.

The two positive egg samples were egg yolk solids from Topeka, Kansas and a non-pasteurized egg-white liquid. The one corn meal containing salmonellae did not contain any egg products, or animal products.

Table 1. The nature and number of specimens examined, and the number of specimens containing salmonellae.

Nature of specimen	Number examined	Number of specimens containing salmonellae
Cake mixes	92	7
Eggs	48	2
Cookie dough	30	0
Biscuits	14	0
Corn meal	10	1
Dinner rolls	2	0
Pie mix	6	0
Tankage	1	0
Horse feed supplement	1	0
Milk replacer and calf starter	1	0
Total	205	10

Three of the cake mixes contained salmonellae of the serotype S. infantis. The rest of the 10 positive samples contained S. muenchen.

The second set of experiments was carried out on the newly identified 10 positive cultures in addition to four more positive cake mixes that were two years old, and had been stored in the refrigerator. They were known to contain S. infantis and S. tennessee.

Two of the cake mixes contained eggs and the other two did not, although they contained some other animal product, like milk.

All the differential plating media used were the same, viz., B.G.A., B.S.A., MacConkey agar and S.S. agar, except in one method where 1.6% solution of sodium sulfadiazine in distilled water was added, resulting in about 8 mg of sodium sulfadiazine per 100 ml of B.G.A. the following steps including transferring of colonies to T.S.I. slants or K.I.A. slants phage typing, and serological typing, with some biochemical tests, are identical in all the methods.

In this work, no emphasis was put on the nature and identification of the contamination. The following table (Table 2) helped in forming an idea about the nature of the contaminant colonies at hand. The table shows the appearance of colonies of the enteric organisms and other common contaminants on four selective plating media that were used in this work.

The words excellent, very good, good, fair, and poor were used to record an idea about the suitability and the degree of success obtained from a particular plating medium used in a particular method. The results of this appraisal are detailed in Table 3.

In addition to the six main methods mentioned, the table includes the results obtained from the screening method using lactose broth preenrichment currently in use by the U. S. F. D. A. which was used first on samples and the positive tests were confirmed by the original North method.

Judging from the appearance of the plates, which took into consideration the colonial distribution in general, the ratio of the salmonellae-like colonies to the contaminant colonies, and the change of the medium, if any, could be observed.

Table 2. Description of the colony appearance of different organisms commonly seen on the four differential plating media.

Genus of organism	Bismuth sulfite	S.S. and MacConkey	Brilliant green
Salmonella typhi	Fully developed colonies are convex 1-3 mm; black with lustrous surface; form a shallow, soft, black pit in medium below colony; immature or overcrowded colonies range from clear, light green, puntiform colonies 1 mm in diameter with black centers by transmitted light. Light green colonies have a darker green center.	After 24 hr colonies usually colorless, transparent, but may have light tan, light pinkish appearance or tan color; 1 to 5 mm in diameter.	Transparent pink colonies. Grows rather sparsely, if at all.
Salmonella group	Colonies similar to above but tending toward a dark rich brown rather than black; usually very lustrous. Colonies usually much larger than S. typhi, frequently dark brownish centers and light edge.	Colonies sometimes larger than above; otherwise similar; sometimes show black centers on S.S.	Transparent pink to deep fuschia colonies. Occasionally will be brownish, showing little change in color or medium. If plate is heavily contaminated with coliform organisms, salmonellae may be masked and appear as transparent green colonies. Close observation reveal the brownish appearance of these colonies.

Table 2. (continued)

Genus of organism	Bismuth sulfite	S.S. and MacConkey	Brilliant green
Arizona group	Colonies similar to Salmonella.	Slow lactose fermenting types similar to salmonellae; rapid lactose fermenting types will resemble coliform colonies.	As on S.S. slow lactose fermenters resemble salmonellae; rapid lactose fermenters are yellowish green similar to coliforms.
Shigella group	Most strains do not grow on this medium although occasional strains of S. flexneri and S. boydii may grow. They appear light to dark green, smooth, flat and glistening.	Colonies usually colorless, transparent; may be smaller than salmonellae. Shigella sonnei may grow especially large with yellow centers and irregular edges.	No significant growth.
Coli-aerogenes group	Greatly inhibited; strains which grow show green to colonies without pitting of medium, may be similar to immature typhoid colonies; yellowish and brick-red colonies are probably members of this group.	Largely inhibited on S.S.; may develop as large, opaque colonies with varying shades of pink or red color throughout. Large mucoid colonies may have pink centers with white or yellow peripheries. Some E. coli O:lll related strains may show transparent colonies on S.S. On MacConkey colonies may be surrounded by a precipitate of bile salts in the medium.	Large opaque yellowish green colonies inhibited to some extent on BGS.

Table 2. (continued)

Genus of organism	Bismuth sulfite	S.S. and MacConkey	Brilliant green
Proteus group	Similar to coliform group. Usually flat, bright to brownish green, with darker centers.	Usually small, transparent water-clear colonies; may have "fuzzy" or veil-like edge. Sometimes show black centers.	Greatly inhibited.
Bethesda group	Some appear as light green colonies with dark green centers flat with entire edges. Some produce greenish brown colonies with dark centers.	Clear transparent colonies similar to salmonellae. Occasionally black centered on S.S.	Usually similar to salmonellae.
Pseudomonas group	Greenish brown colonies sometimes with darker centers. Similar to paracolona.	Transparent grayish colonies usually rough with irregular edges.	Deep pink to purplish colonies usually "fuzzy" edges on BG. On BGS usually inhibited. May develop as pinpoint colonies in 36-48 hours.
Aerobacter group	Medium, circular raised, smooth, entire, brownish, with no change on the medium.	Medium, circular, raised, smooth, entire, brownish to dirty pinkish center with clear zone.	Medium, circular, raised, smooth, entire, yellow with yellow zone; unctuous.

Table 2. (concluded)

Genus of organism	Bismuth sulfite	S.S. and MacConkey	Brilliant green
Citrobacter group	Small, circular, umbonate, smooth entire, greenish brown, with no change on the medium, butyrous.	Small, circular, umbonate, smooth, entire, colorless or dark with clear zone on margin, butyrous.	Small, circular, umbonate, smooth, entire, pink with an intense red zone, butyrous.
Serratia group	Large, circular, convex, smooth, entire, intensely red, no change on medium, butyrous.	Large to medium, circular, convex, smooth, entire, intensely red on MacConkey, light red on S.S. No change on medium, butyrous.	Large, circular, convex, smooth, entire, intensely red, with a light pink zone, butyrous.

Table 3. Colonial distribution, and salmonellae to contaminants ratio, as shown on selective media, using different methods of isolation.

Procedure	Brilliant green agar		MacConkey agar		S.S. agar		Bismuth sulfite agar	
	Colonies	Ratio	Colonies	Ratio	Colonies	Ratio	Colonies	Ratio
Nutrient broth and mannitol purple pre-enrichment methods *	excel-lent; pure culture	excel-lent; pure culture	excel-lent; pure culture	excel-lent; pure culture	excel-lent; pure culture	excel-lent; pure culture	fair to poor	pure culture
Lactose broth[1, 2] pre-enrichment into cystine selenite	very good	high	excel-lent	high	excel-lent	high	fair	equal
Lactose broth pre-enrichment into brilliant green tetra-thionate	good	high	very good	high	excel-lent	high	no growth to poor	no growth to very low
Brilliant green tetrathionate enrichment	good	high	very good	high	not used	not used	no growth to poor	no growth to very low

Table 3. (concluded)

Procedure	Brilliant green agar		MacConkey agar		S.S. agar		Bismuth sulfite agar	
	Colonies	Ratio	Colonies	Ratio	Colonies	Ratio	Colonies	Ratio
Selenite cystine[3] enrichment	fair	high	good	high	fair	relatively high	no growth	no growth
Screening test[1, 2] for salmonellae using lactose broth pre-enrichment	very good	high	excellent	high	very good	high	no growth	very low to no growth

Colonies = Colonial distribution and presence of typical colonies.
Ratio = Ratio of salmonellae to contaminants.

1 = The plates seeded from the highest dilution cultures, were taken into consideration in this table.

2 = In both methods when lactose broth was used, it was limited to the dry positive samples only.

3 = In the selenite cystine enrichment method, the results represent cultures that were incubated for 48 hrs instead of 24 hrs.

* = The first method consists of two separate procedures and is considered as two separate methods in the text.

The mannitol purple and nutrient broth pre-enrichment methods were superior to the lactose broth pre-enrichment which was used for the examination of dried samples only.

Of the two types of lactose broth pre-enrichment, the one using selenite cystine was superior to the one using brilliant green tetrathionate as an enrichment medium.

As far as the two direct enrichment methods are concerned (brilliant green tetrathionate and selenite cystine), the former was superior to the latter.

In the experiment varying the time of incubation of selenite cystine medium from 24 hours at 37 C to 48 hours, better colonial distribution and a higher ratio of salmonellae isolations to contaminants occurred.

The results by the North method were essentially equivalent to those obtained from the screening method using lactose broth pre-enrichments.

Effect of Tergitol No. 7

This experiment was carried out on 10 samples using the two direct enrichment methods (selenite cystine and B.G. tetrathionate) with the addition of Tergitol and without Tergitol. There was no noticeable difference in the results on the plates or later on the tubes.

As can be seen from studying Table 3, bismuth sulfite agar was inhibitory to both salmonellae and contaminants, and throughout the work very few distinct colonies appeared, if any, so the use of the medium was abandoned.

Reactions in Triple Sugar Iron Agar or Kligler Iron Agar

These media were examined after 24 hours incubation and if their appearance suggested contamination they were re-streaked on MacConkey plates for purity. The results were compared with the summary of reactions in Table 4, which gives the probable group depending on the reaction produced. Table 5 shows the results on triple sugar iron agar obtained from the 6 methods tried. Here again the results on the differential tube media were similar to those of the differential selective plating media, giving the same conclusions about the superiority of one method over the other and ultimate selection of the method of choice.

Results on Phage Lysis

Results with the phage lysis test were in complete agreement with the results on the plates and the tubes. Only salmonellae were lysed by the phage.

One suspected culture, however, did lyse with the phage, but on confirmation of the identification, it was found to be an aerogenic coliform.

Results on Serology

Somatic antigenic structure of the salmonellae isolated was determined. The flagellar "H" antigenic configuration, however, was difficult to elucidate, because the sera for the flagellar factors were unavailable and "forced induction" could not be carried out to differentiate phase one and phase two.

Table 4. Reactions on triple sugar iron agar.

Triple sugar iron agar			
Slant	Butt	H_2S	Probable group
Purplish or no change	Alkaline or no change	-	Pseudomonas-like
Alkaline or no change	Alkaline or no change	-	Alcaligenes-like
Acid	Acid and gas	-	Possibly pathogenic strains of E. coli or Klebsiella
Acid	Acid	-	Euterococci and occasionally S. typhi, E. coli, and the dispar type Escherichia may give this reaction; Gram stain recommended
Alkaline spreading growth	Gas	+	Probable Proteus. Confirm with urea test
Acid	Acid and gas	+	Proteus - discard
Alkaline	Acid	± or	Shigella, S. typhi, Proteus or Providence and occasionally other anaerogenic salmonellae
Alkaline	Acid and gas	± or	Salmonellae, Proteus, some Arizona, and some Citrobacter (formerly E. freundii or Bethesda group)

Note: Alk. slant - indicates lactose and sucrose not fermented.
Acid slant - indicates lactose or sucrose fermented.
Alk. butt - indicates dextrose not fermented.
Acid butt - indicates dextrose fermented.

Table 5. Reactions on triple sugar iron agar or Kligler's iron agar, and the probable groups suspected. (Referring to Table 4.)

Procedure	Reactions on T.S.I.A.	Suspected group
Nutrient broth and mannitol purple pre-enrichment methods	All the tubes showed typical salmonellae reactions	Salmonellae
Lactose broth pre-enrichment, using cystine-selenite	95% of the tubes were typical of Salmonella	Salmonellae
Lactose broth pre-enrichment, using brilliant green tetrathionate	About 90% of the tubes were typical	Salmonellae
Selenite cystine enrichment method	Less than 70% of the tubes were typical	Salmonellae
Screening test of salmonellae, using lactose broth pre-enrichment	About 95% of the tubes were typical	Salmonellae

Note: 1. At least 3 Salmonella-like colonies were picked from each plate into T.S.I. agar tubes.

2. In case of the presence of some contaminant tubes, they were referred to as contaminants only, and later confirmed by phage lysis.

3. The above percentages represent the average of tubes inoculated from the 4 different plating media that showed growth.

No agglutination was obtained with any culture that was not a *Salmonella* species.

Referring to the Kauffman-White Scheme in *Serological Identification of the Salmonella* (Difco Laboratories, Detroit, Michigan, U. S. A.) and other serological tables furnished in the index, the following was set as the final identification of the positive samples isolated.

Table 6. Results of the serological examination of the isolates and the results of phage lysis test.

Salmonella serotype	Phage lysis	Somatic "O"	Phase 1	Phase 2
S. tennessee	+	C_1	3; 29	-
S. infantis	+	C_1	r	1.5
S. muenchen	+	C_2	d	1.2

In the case of the four known positive samples, re-running them provided a test for the viability of salmonellae in food materials under refrigeration. The first isolation of salmonellae from these samples was in June, 1962, while the second isolation done in this work was June, 1964. After 2 years of storage, the salmonellae were still viable.

Considering all the facts, data, and information mentioned above, it is proffered that the method of mannitol purple and nutrient broth pre-enrichment is the method of choice for all kinds of foodstuffs including moist and dry, while the lactose broth pre-enrichment is limited to dry foodstuffs. Regarding direct enrichment methods, the brilliant green

tetrathionate medium was superior to selenite cystine. The screening method using lactose broth pre-enrichment produced results that were in full agreement with the North method. The use of brilliant green tetrathionate medium subsequent to the lactose broth pre-enrichment method instead of selenite cystine was inferior to the regular "North method" of isolation.

DISCUSSION

In any kind of research the choice of the proper methods is an essential step in the success of that work. This is true in the case of the isolation of salmonellae from food products. The proper choice of media and procedures is a great determining factor in the efficiency of recovery of salmonellae from foods.

Workers on this subject have always been faced with certain difficulties and disadvantages, the most important of which is the fact that most of the media and procedures available and actually in practice have generally been adapted from procedures meant for the isolation of salmonellae from clinical specimens and not from food.

Therefore, the aim of every worker in this field is to search for new methods that are more or less specific for the work with foods, or at least study the methods that are in use and try to develop, modify, and consequently choose the best of them and employ them for the isolation of salmonellae from food. Again, the old problem of the need of special, different methods for every kind of food product arises and presents another factor to be considered and evaluated.

Another problem that needs to be considered is the fact that most of these food products contain organisms that have suffered a reduction in their activity due to different kinds of processing during manufacture, and consequently need to be stimulated by pre-enrichment media to build up their population before they can be transferred to selective media. With that aim in mind, the three pre-enrichment methods in this study (nutrient broth, mannitol purple, and lactose broth pre-enrichment) were put to use and compared.

In all cases the choice of media and procedures was made after careful consideration of the available data and information obtained from a review of the literature. This included the effect of the presence of large amounts of organic matter in the sample on the performance of selective enrichment media. Pre-enrichment broth was found by many workers to give better results than reconstitution of materials in distilled water. The quality of peptone as found by North and Bartram (1953) affected the productivity of selenite "F" medium. The use of small amounts of yeast extract remedied the defect. Therefore, the use of the two non-inhibitory media nutrient broth and mannitol purple medium was recommended. In the first medium proteose peptone No. 3 (Difco) was used, yeast extract and beef extract were also added to provide essential growth factors. In the second medium mannitol was added to provide a fermentable substrate usually utilized by salmonellae. This was an advantage over nutrient broth because the mannitol facilitated active multiplication of salmonellae even if they occurred in small numbers.

The adverse effect of the presence of excessive amounts of organic matter (Taylor and Galton, 1958; 1961) was corrected by adding a 10%

suspension of the sample to the pre-enrichment medium, then transferring only 1 ml of the mixture after incubation. Incubation was 12 to 15 hrs, which provided the best time for the highest population. After pre-enrichment four selective media were used with the idea that if one organism is inhibited by one medium there will always be a chance that it might not be inhibited by another medium. Tetrathionate was modified by the addition of brilliant green according to Osborne and Stokes' report (1955) that brilliant green is effective in limiting growth of the contaminating organisms.

Cystine and sulfapyridine were added to selenite "F" because of further reports on good results obtained by North and Bartram (1953) and Osborne and Stokes (1955). Generally it was thought that the combination of both pre-enrichment and selective media could complement and supplement each other, resulting in maximum recovery.

Previous results and the observations gained from this work showed that action of both pre-enrichment media was almost identical except for the fact that colonial distribution of cultures originating from mannitol purple broth was better than that originating from nutrient broth. This might be attributed to the fact that nutrient broth allowed both salmonellae and contaminants to grow equally well, while in mannitol purple the utilization of mannitol by salmonellae could result in the latter outgrowing the contaminants. In addition, utilization of mannitol could result in enough acidity due to fermentation to check the growth of contaminants. The same is true in the case of the use of lactose broth pre-enrichment. North (1961) confirmed the superiority of this procedure to direct transfer of material into selective media. It was stated that

utilization of lactose by salmonellae and its consequent fermentation could lower the pH sufficiently to inhibit other organisms without any apparent adverse effect on salmonellae.

Observations in this and other work showed that the numbers of salmonellae colonies compared to the contaminant colonies, as well as the uniform distribution of these colonies on the plates, were superior in those cultures originating from selenite cystine sulfapyridine, and brilliant green tetrathionate, than in unmodified selenite "F" and tetrathionate, respectively. This was explained as due to the fact that both brilliant green and tetrathionate added to the selective media, are essentially inhibitory to the contaminants, while the unmodified selenite "F" and tetrathionate which were lacking in these inhibitory substances, supported the growth of contaminants more than the modified media.

Results and data obtained by Banwart and Ayres (1953) showed the inhibitory effect of some of the plating media on certain strains of salmonellae while other media did not. Therefore, the most suitable thing to do was to use as many plating media as possible to give a better chance for recovery. Generally speaking, brilliant green agar was more advantageous than others in this respect. Bismuth sulfite agar was thought to be good but results and experience with it in this study showed that it was very inhibitory to both salmonellae and contaminants. S.S. agar was thought to be highly inhibitory but its use on a considerable number of samples in this study showed that it was as good as MacConkey's agar, which was thought to be the least selective by Edwards and Ewing

(1962). In this work MacConkey's agar proved to be as good in this respect as brilliant green agar.

Edwards and Fife (1961) recommended bismuth sulfite agar plates for the detection of Arizona strains, as they are slow lactose fermenters and can be detected on the brilliant green sulfapyridine plates. However, other organisms that have also been found in food and in cases of food infection ferment lactose rapidly and could be missed. Both Arizona strains and salmonellae produce similar typical black colonies on bismuth sulfite agar, and they have the common ability to produce both H_2S and lysine decarboxylase, not shared by other enteric bacteria. To determine this a lysine iron agar was developed to be inoculated from black colonies on bismuth sulfite agar. By this method rapid lactose-fermenting Arizona could be differentiated from salmonellae, but we did not use this plate in our study because as was mentioned before, the bismuth sulfite agar was not satisfactory, due to its high inhibitory effect. This fact was more intensified in the case of the nutrient broth - selenite "F" combination which though it had a definite inhibiting effect on contaminants, one noticed that it suppressed the growth of salmonellae as well.

It was obvious from the colonial distribution on differential plates that selenite "F" and tetrathionate were identical as far as their use in isolation.

Taylor (1957) used selenite medium as an enrichment medium in isolation of salmonellae from eggs, after the addition of cystine and the substitution of lactose with mannitol or dulcitol, with the idea that those two carbohydrates are utilized by salmonellae while lactose is not. This

sounded quite logical, but Leifson (1936) claimed that change of carbohydrate did not produce any effect over lactose, and that the mere function of the carbohydrates is to reduce the over-growth of coliforms or enterococci by offering a source of acid to prevent the pH of the medium from rising during the bacterial growth, and consequently the loss of selenite toxicity in an alkaline pH range.

Wells, Bergquist and Forsyth (1957) suggested a new modification of selenite broth by substituting lactose with mannitol and adding bile salts and brilliant green as additional selective agents besides sodium selenite. They claimed its effectiveness in preventing the growth of Escherichia and Proteus, but here this modification was not used because the selenite cystine medium was quite effective in that respect.

Experience with pure egg products showed that the brilliant green tetrathionate method was superior to selenite cystine enrichment method. These results do not agree with those reported by Dack (1956) who worked on egg albumen and found that the greatest number of salmonellae cultures was obtained from selenite "F" with added cystine. He also found that bismuth sulfite plating medium yielded more salmonellae cultures than brilliant green agar from both non-pasteurized and pasteurized liquid eggs, and that the two plating media were equally effective for isolating salmonellae from pasteurized glucose-free egg powder. These results disagree with the experiments reported here with bismuth sulfite, which proved very unsatisfactory as mentioned before.

It has been shown that selenite broth effectively selects salmonellae, and inhibits closely related Gram negative bacteria such as Pseudomonas, Proteus and coliforms. Sodium selenite present in the broth is toxic to

enterobacteria, but the ability of salmonellae to grow in selenite appears to be due to binding of selenite by constituents of peptone-like peptides. Reduction of selenite takes place after the growth is established and the intensity of reduction is related to the profuseness of growth.

Selectivity in selenite appears to be associated with sulphur metabolism, and cystine was found to be one of the most preferred sulphur sources by bacteria. Therefore, it was added to selenite to encourage the growth of these bacteria, including salmonellae. Another of cystine's properties is the annulling of the toxicity of selenite. North and Bartram (1953) discovered this property when addition of cystine enhanced the growth of S. typhimurium. The amount of cystine added was very small. Growth was stimulated by as little as 0.004 microgram per 100 ml at 0.25% phosphate concentration and reached a maximum at 0.25 microgram per 100 ml. The addition of 1 microgram cystine per 100 ml and reduction in phosphate content to 0.25% was useful in correcting deficiencies in selenite medium. Their data also showed that with a phosphate concentration of 1% the addition of 10 micrograms per 100 ml of cystine resulted in greatly improved productivity.

In this work, however, an already prepared cystine selenite medium was used.

Another defect in selenite medium arose from the lack in the nutritional factors in the kind of peptone used. Selenite also reduces the number of viable cells during the initial incubation period, which presents a very serious problem in samples with a low count of salmonellae.

The lack of inhibition of Proteus and the delay of growth of Escherichia for only a few hours is another disadvantage of selenite broth. Stokes and Osborne (1955) solved this problem by the use of their modified selenite brilliant green medium.

The above disadvantages have a direct effect on the inferiority of the selenite enrichment method to other methods used in this work. No significant improvement in isolation was detected by using Tergitol because all the samples examined did not have a high concentration of fat that needed to be dispersed or emulsified.

As far as the lactose broth pre-enrichment procedure is concerned, many workers engaged in this field (North and Williams, 1960) have found that pre-enrichment is a non-inhibitory medium prior to employment of a selective broth increased recovery of salmonellae from egg samples. Taylor and Silliker (1961) indicated that the microflora of the food is an important determinant in the selection of the proper method of enrichment.

Apparently, two factors confound the selective isolation of salmonellae from food products: (1) physiological dormancy of salmonellae, and (2) the adverse effect of soluble food components on the selectivity of the enrichment media. Dormancy was treated by pre-enrichment in a non-inhibitory media.

Although lactose pre-enrichment is meant to activate physiologically dormant cells, it has a limited applicability because it enhances recovery of salmonellae only under extremely limited coliform-salmonellae ratios. It is still far more sensitive than direct inoculation of either

tetrathionate or selenite broths, especially for dried egg albumen and other dried food samples.

In spite of all the work done so far on salmonellae in food, a good method for isolation is still lacking. Dack (1956) claimed that several people in medical science during a trial of court cases have stated under oath that current methods for demonstrating salmonellae could be faulty and that negative results on samples of food, or carrier's materials, are not conclusive evidence of the absence of salmonellae.

SUMMARY

Isolation of salmonellae from 10 kinds of foodstuffs was attempted with 7 methods of isolation.

A total of 205 samples was examined, 10 of which were proven to contain salmonellae.

Fourteen samples known to contain salmonellae were re-examined using the same six methods. Comparison between these procedures was based on their superiority of producing the best isolations of salmonellae from naturally contaminated food materials.

Of the 4 pre-enrichment procedures employed, the mannitol purple and nutrient broth pre-enrichment methods were the best.

The lactose broth pre-enrichment method was superior to the two direct enrichment methods (selenite cystine and brilliant green tetrathionate). Brilliant green tetrathionate was in turn superior to selenite cystine medium and the lactose broth pre-enrichment into brilliant green tetrathionate medium was inferior to lactose broth into selenite cystine.

The screening test for salmonellae employing lactose broth pre-enrichment produced results that were almost identical to the regular lactose broth pre-enrichment procedure, or the North method.

Several other experiments were carried out, including: (1) the effect of Tergitol No. 7; (2) the effect of a longer period of incubation of selenite cystine broth cultures; and (3) a viability test using two-year-old Salmonella-positive samples. The first proved to be of no great significance. The second showed a considerable improvement. The third proved that salmonellae can stay viable under refrigeration conditions for a long time (two years in this case).

ACKNOWLEDGMENTS

The author expresses gratitude, appreciation and respect to his major adviser, Professor V. D. Foltz, for his guidance, advice and encouragement throughout the course of this work.

He is especially grateful to Dr. T. H. Lord for all the help he has given and for reading the manuscript.

Thanks are also due Mr. R. W. Schrader for supplying some of his data that the author used in this work.

BIBLIOGRAPHY

Adinarayanan, N. and V. D. Foltz. 1963.
Dept. of Bacteriology, Kansas State University. Personal communication.

Akiyama, R. S. 1961.
Studies on Salmonella infection in chicken. Japanese J. Bact. 16(5):430-435.

Akopian, R. A. 1960.
An outbreak of food poisoning caused by S. paratyphi (translated title). Microbiol. Epidemiol. and Immunobiol. 31(12):2301-2302.

Angelotti, Robert. 1961.
S. infantis isolated from ham in food poisoning incident. Publ. Health Rept. 76(a):711-776.

APHA, 1955.
Am. Public Health Association, Standard method of examination of sewage and industrial waste. 10th Ed., New York, New York.

Banwart, C. J. and John Ayres. 1953.
A study of the effect of broth and agar on salmonellae growth. Enrichment broth and selective agar. App. Microbiol. 1:296-301.

Byrne, A., G. Morton, L. G. Rayman, R. L. Morris and P. Schneider. 1955.
Methods of detection and estimation of the number of salmonellae in dried egg and other food products. Appl. Microbiol. 3:368-372.

Brede, L. M. 1963.
A new Salmonella in feces of South African infants, known as Salmonella kuilsrivier. J. Hyg. 10(1):115-116.

Cherry, W. B. and P. R. Edwards. 1954.
A simple procedure for identification of the genus Salmonella by specific bacteriophage. J. Lab. and Clinic Med. 44:51-55.

Dack, G. M. 1956.
Symposium on Methodology for Salmonella in food. Bact. Rev. 19: 275-276.

De Capito, Thelma. 1963.
Isolation of salmonellae from flies. Am. J. Trop. Med. and Hyg. 12:#892, #1963.

Dung, D. S. and G. F. Kang. 1963.
An attempt to use bacteriophage in typing of Salmonella choleraesuis. Acta. Microbiol. Sinica. 9(1):102-104.

Edwards, P. R. and D. W. Brunner. 1947.
The genus Salmonella: its occurrence and distribution in the United States. Ky. Agr. Exp. Sta. Bull. 525.

Edwards, P. R. and W. H. Ewing. 1962.
Identification of enterobacteriacae. Burgess Publishing Co., Minneapolis 15, Minnesota.

Edwards, P. R. and M. A. Fife. 1961.
Lysine iron agar in the detection of Arizona cultures. App. Microbiol. 9:478-480.

Ellingsen, G. 1962.
Salmonella in shredded cocoanut. Am. J. Trop. Med. and Hyg. 14:24-38.

Essex, Cater, A. J. Jones, and F. Swindell. 1963.
An outbreak of human Salmonella infection, due to typhimurium phage-4-associated with the use of an unpasteurized liquid egg. J. Hyg. 61-3:323-330.

Francis, D. W. 1963.
A study of Salmonella infection in a flock of chukar partridges. Avian Dis. 7(4):501-506.

Frankl, William and Fred Rogers. 1961.
Salmonellosis due to contaminated fish. Tram. and Stud. Coll. Physicians, Philadelphia. 29(2:86-88.

Galton, M. M., J. R. Boring and W. T. Martin. 1964.
Salmonellae in food (a review of methods for isolation and a suggested procedure). U. S. Dept. of HEW. Public Health Service. Communicable Disease Center, Atlanta, Georgia.

Galton, M. M., M. Horless and A. J. Hardy. 1953.
Salmonellae isolation from dehydrated dog meals. J. Am. Vet. Med. Association 126:57-58.

Galton, M. M., J. E. Scatterday and A. V. Hardy. 1963.
Salmonellosis in dogs. J. Inf. Dis. 91:1-18.

Galton, M. M., W. D. Lowery and A. V. Hardy. 1954.
Salmonellae in fresh and smoked sausage. J. Inf. Dis. 96:232-235.

Gibbons, N. E. and R. L. Moore. 1944.
Dried whole egg powder (occurrence and distribution of Salmonellae). Can. J. Res. 22:48-57.

Guinee, P. M. 1961.
Three new types of Salmonella. J. Microbiol. and Serol. 27(4): 465-468.

Haas, J. 1962.
The frequency of occurrence of Salmonella in egg products. Wiener Tierarzh. Monatsschr 49(12) - 1962.

Jameson, J. E. 1961.
A study of tetrathionate enrichment techniques with particular reference to two new tetrathionate modifications used in isolating Salmonellae from sewer swabs. J. Hyg. 59:1-13.

Jansen, L. B. 1955.
Symposium on methods of Salmonellae in food. Bact. Rev. 191:275-276.

Joshi, L. M. 1963.
Bacteriophage typing of S. paratyphi "A". J. Post Grad. Med. 9(3):115-120.

Khakareva, T. P. 1963.
Oranienburg Salmonellosis in the region of Corkii. (Translated title.) Zhur. Microbiol, Epidimiol, and Immunobiol. 40(6):128-130.

Killesso, V. A. 1963.
Identification of Salmonellae with the aid of bacteriophage. (Translated title.) Zhur. Mikrobiol. Epidimiol. and Immunobiol. 40(7): 86-89.

Leifson, E. 1936.
New Salmonella enrichment medium for the isolation of typhoid and paratyphoid bacilli. Am. J. Hyg. 24:423-432.

LeNoc, P. S. 1963.
Salmonellae isolated in Madagascar up until 1962. Arch. Inst. Pasteur. 31(2):101-106.

Lerche, M. 1961.
The viability of Salmonellae in mayonnaise and meat salad. Wiener Tierazh. Monatsschr. 48(6):348-361.

Levin, Hugh S. 1961.
Salmonella pericarditis. Ann. Interval Med. 55(5):817-823.

McCullough, Norman B. and Anne F. Byrne. 1952.
Relative efficiency of different culture media in isolation of certain members of the Salmonella group. J. Inf. Dis. 90:71-75.

Makholia, B. D. 1963.
Salmonella vrindaban, a new anatypical serotype from pigs. Internat. Bull. Bacteriol. 13(4):177-178.

Manten, R. O. 1963.
Incidence of resistance to chloramphenicol and tetracycline among 13502 Salmonella strains isolated in 1961. J. Microbiol. and Serology 28(4):428-434.

Marcus, B. W. 1963.
The incidence of Salmonella in West Berlin. Hyg. 184(2):147-161.

Martin, Hyres. 1961.
The isolation of intestinal pathogens by selective media. J. Path. and Bact. (54):193-207.

Milch, Hedda, and Vera G. Laizlo. 1963.
Supplemental phage typing with Salmonella typhimurium and its use in epidemiology. Acta. Microbiol. Acad. Sci. Hung. 10(1):41-52.

Montford, Jeannine and F. S. Thatcher. 1961.
Comparison of four methods of isolating salmonellae from food and elaboration of preferred method. J. Food Sci. 26(5):510-517.

Muller, J. 1957.
The Salmonella problem in Denmark. Bull. Office Internatl. Epizootien. 48:323-333.

North, William R. 1961.
Lactose pre-enrichment method for isolation of salmonellae from dried egg albumen. Appl. Microbiol. 9:188-195.

North, W. R. and M. T. Bartram. 1953.
The efficiency of selenite broth of different compositions in the isolation of salmonellae. Appl. Microbiol. 1:130-135.

Osborne, W. W. and J. L. Stokes. 1955.
A modified selenite B.G. medium for isolation of salmonellae from eggs. Appl. Microbiol. 3:295-299.

Pollo, Antolin and F. G. Mellande. 1960.
Salmonella in handled meat. Rev. Sanidad. Hig. Publ. 34(7/8/9): 488-535.

Ratna, Lie and C. E. Dollman. 1948.
Experience with Salmonella typing in Canada. Can. J. Public Health 38:280-294.

Richardson, C. K. 1961.
Salmonella in a group of periurban South African school children. J. Hyg. 61(2):257-263.

Sachideva, L. D. 1963.
Meningitis due to Salmonella virchow in an infant. Indian J. of Path. Bact. 6(3):134-141.

Sharma, V. K. 1962.
Phage type of S. typhimurium from man and animal. 5th Indian Microbiological Congress. Indian J. Microbiol. 2(3):141-142.

Sharma, V. K. 1962.
Phage typing of S. typhimurium isolated from human and animals. Indian J. Microbiol. 2(4):157-160.

Sharma, V. K. 1963.
Salmonella serotypes from human carrier. J. Med. Res. 51(3): 399-401.

Sharma, V. K. 1963a.
S. brijbhumi, a new serotype from sewage. Internatl. Bull. Bact. Nomen. and Taxonomy 13(4):179-180.

Sharma, V. K. 1963b.
Salmonella serotypes from sewage. Indian J. Med. Res. 151(3):1-5.

Silberman, D. L. 1963.
Primary mycotic aneurysm of aorta . . . a complication of salmonellosis. Angiology 14(7):372-376.

Silliker, J. H. and W. I. Taylor. 1958.
Isolation of salmonellae from food samples. II. The effect of added food samples upon the performance of enrichment broths. Appl. Microbiol. 6:228-232.

Silliker, J. H., R. H. Deibel, and P. T. Fagan. 1964.
Enhancing effect of feces on the isolation of salmonellae from selenite broth. Appl. Microbiol. 12:100-105.

Silliker, J. H., R. H. Deibel and P. T. Fagan. 1964.
Comparison of methods of salmonellae isolation from egg products. Appl. Microbiol. 12-224.

Slocum, G. G. 1955.
Symposium on methodology of Salmonella in food. Bact. Rev. 19:275-276.

Smith, H. G. 1952.
Evaluation of culture media for the isolation of salmonellae from feces. J. Hyg. 50:21-36.

Smith, H. G. 1959a.
Isolation of Salmonella for mesenteric lymph nodes and feces of pigs, cattle, sheep, dogs, cats and poultry. J. Hyg. 57:266-273.

Smith, H. G. 1959b.
The nature of selective action of selenite broth. J. General Microbiology 21:61-71.

Smyser, C. F. 1963.
Detection of S. typhimurium from artificially contaminated poultry feed and animal by-products. Avian Dis. 7(4):423-434.

Stephens, J. F. 1963.
Concurrent Salmonella typhimurium infection in chicks. 52nd Annual Meeting of Poultry Science Association. Poultry Sci. 42(5):1310.

Stokes, J. L. and W. W. Osborne. 1955.
A selenite medium for the isolation of salmonellae. Appl. Microbiol. 3:217-220.

Taylor, W. I. and J. H. Silliker. 1958.
Dulcitol lactose iron agar. A new differential medium for confirmation of the genus Salmonella. Appl. Microbiol. 6:335-338.

Taylor, W. I. and J. H. Silliker. 1961.
Isolation of salmonellae from food samples. IV. Comparison of methods of enrichment. Appl. Microbiol. 9:484-486.

Thatcher, F. S. 1963.
The microbiology of special frozen foods in relation to public health. J. Appl. Bacteriol. 26(2):266-285.

Thatcher, F. S. and J. Montford. 1962.
Egg products as a source of Salmonella in processed food. Canadian J. of Public Health 52(2):61-69.

Thomas, J. 1961.
Salmonella isolated from animal and food products of animal origin in Belgium. Ann. Med. Vet. 105(4):206-218.

Thomson, S. 1955.
The number of pathogenic bacilli in feces in intestinal disease. J. Hyg. 53:217-224.

Triozon, F. 1963.
A small epidemic of paratyphoid B. in newborns. Ann - pharmacent France 21(2):180.

Van Eck, E. A. 1961.
The isolation of salmonellae from clinical specimens not usually associated with Salmonella etiology. Indian J. of Path. and Bact. 4(2):70-77.

Wells, F. Bergquist and R. H. Forsythe. 1957.
A comparison of selective media, for isolation of salmonellae, from commercial egg white solids. Appl. Microbiol. 6:198-201.

William, Clog. 1961.
Two cases of meningitis caused by S. eberth bacilli, in infants. Press. Med. 69(38):1663.

Wilson, Elizabeth. 1961.
Prevalence of salmonellae in meat and poultry products.
J. Inf. Dis. 109(2):166-171.

Wright, M. L. and G. W. Anderson. 1962.
Salmonella isolation from food additives of animal origin.
Lab. section, Canadian J. Publ. Health 53(1):36.

APPENDIX

Table 7. Bacto-Salmonella O antisera and antigens with which each reacts.

Code	Bacto-Salmonella O antisera	Identifying antigens	Other antigens
2814	Salmonella O Antiserum Group A	2	
2815	Salmonella O Antiserum Group B	4	5
2816	Salmonella O Antiserum Group C_1	7	
2817	Salmonella O Antiserum Group C_2	8	
2818	Salmonella O Antiserum Group D	9	
2257	Salmonella O Antiserum Group E_1	10	
2258	Salmonella O Antiserum Group E_2	15	
2259	Salmonella O Antiserum Group E_4	19	
2260	Salmonella O Antiserum Group F	11	
2261	Salmonella O Antiserum Group G	13	22, 23
2262	Salmonella O Antiserum Group H	14	24, 25
2263	Salmonella O Antiserum Group I	16	
2827	Salmonella V_1 Antiserum		
2264	Salmonella O Antiserum Poly A-1 Poly contains agglutinins for Salmonella Groups A through I and V_1	2, 4, 7, 8, 9 10, 15, 19, 11, 13, 14, 16	1, 2, 3, 5, 6 10, 12, 22, 23, 24, 25
2892	Salmonella O Antisera Set Contains 1 vial each of group A, B, C_1, C_2, D, E_1, E_2, E_4, F, G, H, I, Poly A-1 and V_1		

Table 8. H antisera selection.

Cultures positive with O antiserum:	Should be tested with H antisera: Phase 1	Phase 2
A	a, G complex	
B	a, b, c, d, eh, G complex, i, k, L complex, r, y, z, z_4 complex, z_{10}, z_{29}	en complex L complex I complex
C_1	a, b, c, d, eh, G complex, i, k, L complex, r, y, z, z_4 complex, z_{10}, z_{29}	en complex L complex 1 complex
C_2	a, b, c, d, eh, G complex, i, k, L complex, r, y, z_4 complex, z_{10}, z_{29}	en complex L complex 1 complex
D	a, b, c, d, eh, G complex, i, k, L complex, r, z, z_4 complex, z_{10}, z_{29}	en complex 1 complex
E_1	a, b, c, d, eh, G complex, i, k, L complex, r, y, z, z_4 complex, z_{10}, z_{29}	en complex L complex 1 complex
E_2	d, eh, G complex, L complex, r, y, z_{10}	L complex 1 complex
E_4	a, b, d, G complex, i, L complex r, y, z, z_{10}	en complex L complex 1 complex
F	a, b, d, eh, G complex, i, k, L complex, r, y, z_{10}	en complex L complex 1 complex
G	b, d, eh, G complex, i, y, z, z_4 complex, z_{10}	en complex L complex 1 complex
G	b, d, eh, G complex, i, y, z, z_4 complex, z_{10}	en complex L complex 1 complex
H	a, d, eh, G complex, i, k, L complex, r, y, z, z_4 complex	en complex 1 complex
I	a, b, c, d, eh, G complex, i, k, L complex, y, z_4 complex, z_{10}, z_{29}	en complex 1 complex

Table 9. Bacto-Salmonella H antisera and antigens with which each reacts.

Code	Bacto-Salmonella H antisera	Identifying antigens	Other antigens
2820	Salmonella H Antiserum Group a	a	
2821	Salmonella H Antiserum Group b	b	
2822	Salmonella H Antiserum Group c	c	
2823	Salmonella H Antiserum Group d	d	
2273	Salmonella H Antiserum Group eh	eh	
2270	Salmonella H Antiserum en complex	en	enx, enz$_{15}$
2269	Salmonella H Antiserum G complex	g	fg; fgt; gm; gms; gmt; gp; gpu; gq; gst; mst; mt
2824	Salmonella H Antiserum Group i	i	
2274	Salmonella H Antiserum Group k	k	
2271	Salmonella H Antiserum Group L complex	l	lv; lw; lz$_{13}$
2275	Salmonella H Antiserum Group r	r	
2276	Salmonella H Antiserum Group y	y	
2277	Salmonella H Antiserum Group z	z	
2278	Salmonella H Antiserum Group z$_4$	z$_4$	z$_4$; z$_{23}$; z$_4$z$_{24}$; z$_4$z$_{32}$
2279	Salmonella H Antiserum Group z$_{10}$	z$_{10}$	
2280	Salmonella H Antiserum Group z$_{29}$	z$_{29}$	
2272	Salmonella H Antiserum Group 1 complex	1	1, 2; 1, 5; 1, 6; 1, 7
2893	Salmonella H Antisera Set 1 vial each Group a, b, c, d, eh, i, k, r, y, z, z$_4$, z$_{10}$, z$_{29}$, and en, G, L & 1 complexes		

STUDIES OF THE PROCEDURES FOR THE ISOLATION OF SALMONELLAE FROM FOODS

by

SAMIR A. AL-DAHHAN

D.V.M., Baghdad University, Baghdad, Iraq, 1961

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1965

The work on this study was done in two stages. First, seven methods for the isolation of salmonellae from food were used in examining 205 samples of food; 10 of them were found to contain salmonellae. Only one serotype of salmonellae was isolated from each sample.

S. tennessee, S. infantis and S. muenchen were the serotypes identified from seven samples of cake mixes, two samples of eggs (non-pasteurized egg white and egg yolk solids) and one corn meal.

The second stage was to re-examine 14 samples known to contain salmonellae and use them in comparing the same seven methods used in the study.

Mannitol purple and nutrient broth pre-enrichment procedures, lactose broth pre-enrichment into selenite cystine medium, and lactose broth pre-enrichment into brilliant green tetrathionate medium were the four pre-enrichment procedures used.

The first two were found to be the best, in which cultures were transferred from mannitol purple and nutrient broth to brilliant green, MacConkey, S.S. and bismuth sulfite agar plating media. Those media and the triple sugar iron agar or Kligler's iron agar into which the colonies from the plates were picked, were used in all seven methods. Brilliant green was found to be the most advantageous while bismuth sulfite agar proved to be unsatisfactory due to its high inhibitory action on both salmonellae and contaminants.

The lactose broth pre-enrichment method was found to be superior to both selenite cystine and brilliant green tetrathionate enrichment procedures, while brilliant green tetrathionate broth was superior to selenite

cystine medium, and the lactose broth pre-enrichment into selenite cystine medium was found superior to lactose broth pre-enrichment into brilliant green tetrathionate.

The genus specific Salmonella "O-1" phage was used all through the study as a fast and easy way of early detection of salmonellae cultures, and it spared many, long and complicated biochemical tests. It was highly specific for salmonellae.

Serological identification of phage positive cultures was carried using the somatic "O" antigen agglutination test by which the specific salmonellae groups were identified.

The final serotype identification was done by the Kansas State Department of Health, Division of Public Health Laboratories, Topeka, Kansas.

The effect of Tergitol No. 7 on isolation of salmonellae was studied and found to be of no great significance due to the nature of the samples used. None of them contained any excessive amounts of fat that needed to be emulsified.

The incubation time of selenite cystine broth was lengthened from 24 to 48 hours at 37 C and was found to produce a considerable improvement on the colonial distributions and the ratio of salmonellae colonies to contaminants.